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ANIMAL AND PLANT HEALTH INSPECTION SERVICE
NATIONAL VETERINARY SERVICES LABORATORIES
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SAM 613

		Erysipelothrix rhusiopathiae
9CFR 113.XXX	April 7, 1994	Bacterins
Standard requirement	Draft	

SUPPLEMENTAL ASSAY METHOD

FOR

POTENCY TESTING OF

ERYSIPELOTHRIX RHUSIOPATHIAE BACTERINS

A. SUMMARY

This is an *in vitro* assay method using a direct enzyme-linked immunosorbent assay (ELISA) system to measure the relative potency of bacterins containing *Erysipelothrix rhusiopathiae*.

B. MATERIALS

1. Potassium phosphate elution buffer

KH_2PO_4	8.2 g
Deionized water	q.s. to 100 ml
pH to 6.5	

2. Bacterins containing *E. rhusiopathiae* antigen

   a. Reference bacterin

   b. Test serial(s)

3. Phosphate buffered saline, pH 7.2 (PBS)

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Deionized water	q.s. to 1000 ml

- Adjust pH to 7.2 with 0.1 M NaOH or 0.1 M HCl

4. 0.5% sodium desoxycholate

PBS, pH 7.2	100 ml
Sodium desoxycholate	0.50 g

(Difco Cat. No. 02248-13, Sigma D-6750, or equivalent)

- Store at 4°C up to 30 days; warm to 22°C prior to use

5. Antigen coating buffer (0.5 M carbonate buffer, pH 9.6)

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Deionized water	q.s. to 1 liter

- Store at 4°C for up to 1 week

6. Sodium citrate ($Na_3C_6H_5O_7 \cdot 2\ H_2O$; F.W. 294.10)

7. Wash solution (phosphate buffered saline, pH 7.2, .05% Tween 20)

Tween 20	0.5 ml
PBS, pH 7.2	1000 ml

8. Blocker solution (Use 1 or 2)

a. 5% nonfat dry milk (NFDM)

Nonfat dry milk	5.0 g
PBS, pH 7.2	100 ml

- Make fresh daily

b. 1% Polyvinyl alcohol (PVA)

PVA (80% hydrolyzed, 13,000 MW)	1.0 g
PBS, pH 7.2	100 ml

- Make fresh daily. Store at room temperature (20-25°C)

9. Antibodies

a. *E. rhusiopathiae* monoclonal antibody (MAb) ERHU1-B60-91 in the form of ascites fluid obtained from the National Veterinary Services Laboratories (NVSL). The MAb is stored at -20°C.

b. Conjugate is goat anti-mouse IgG horseradish-peroxidase (HRP) labelled antibody (Kirkegaard & Perry Laboratories, Inc., 2 Cessna Court, Gaithersburg, MD 20879, #04-18-06 or equivalent). Alternately, donkey anti-mouse IgG HRP labelled antibody (Jackson Immunoresearch Laboratories, Inc., PO Box 9, Westgrove, PA 19390, #715-0360151 or equivalent) may be used.

10. Substrate (Use 1 or 2)

a. 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories, Inc., 2 Cessna Court, Gaithersburg, MD 20879, #50-76-00 or equivalent).

b. 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) (Kirkegaard & Perry Laboratories, Inc., 2 Cessna Court, Gaithersburg, MD 20879, #50-62-00 or equivalent).

11. Stop solution for TMB

2.5 M H_2SO_4

C. PROCEDURES

1. Preparation of the bacterin(s)

The reference bacterin and test serial bacterin(s) are treated identically. Bacterin containers are shaken by hand or vortexed to thoroughly mix the contents. Some products may be tested prior to elution. Other products may require elution. Products are eluted as necessary using one of the following elution methods:

a. Potassium-phosphate elution

A 5.0-ml aliquot of bacterin is immersed in an ice water bath and sonicated for 1 minute at a duty cycle of 40 at 20 mhz with a microtip using a high intensity 50-watt ultrasonic processor. The sonicated bacterins are diluted in an equal volume of potassium phosphate elution buffer, and the mixtures are

incubated at 37°C overnight (12-18 hours) on a rotary shaker at 80-90 rpm.

b. Freezing-centrifugation elution

For those samples that are not satisfactorily eluted using the potassium-phosphate elution method, at least 5.0 ml of bacterin is frozen at -70°C for at least 24 hours. Bacterins are thawed at 37°C, and 5.0 ml bacterin is combined with 5.0 ml sodium desoxycholate. A 1-g amount of sodium citrate is added. The mixture is vortexed briefly to dissolve sodium citrate and incubated at room temperature (22°C ± 5°) on a rotary shaker at 80-90 rpm for 30 minutes. The mixture is vortexed briefly, and incubation is continued an additional 30 minutes. The mixture is centrifuged at 500 X g for 10 minutes at 4°C. The supernatant is discarded, and the pellet is resuspended in 5.0 ml potassium phosphate elution buffer. The mixture is immersed in an ice water bath and sonicated for 1 minute at a duty cycle of 40 at 20 mhz with a microtip using a high intensity 50-watt ultrasonic processor. The mixture is incubated overnight (12-18 hours) at 37°C on a rotary shaker at 80-90 rpm.

2. ELISA procedure

a. Untreated or eluted bacterins are sonicated for 1 minute at a duty cycle of 40 at 20 mhz with a microtip using a high intensity 50-watt ultrasonic processor.

b. A 100-μl amount of antigen coating buffer is placed in each well of an ELISA grade 96-well flat-bottom microtitration plate (Immunlon-2, Dynatech Laboratories, Inc., 14340 Sullyfield Circle, Chantilly, VA 22021, or equivalent). A 100-μl amount of the eluted bacterin is placed in column 1. At least two replications of the reference bacterin and of each test serial are tested on the same plate. The last well of each column containing the reference bacterin contains only antigen coating buffer instead of bacterin and serves as a blank. Serial twofold dilutions are made by transferring 100 μl from well to well in columns 1 through 12, except in the reference bacterin columns, which are diluted columns 1 through 11.

c. The plate is incubated at 37°C for 1 hour ± 15 minutes.

d. An automatic plate washer or equivalent is used to aspirate all wells of the plate. The plate is washed three times with 300 μl wash solution/well.

e. A 300-μl amount of blocker solution is added to each well. The plate is incubated at 37°C for 1 hour ± 15 minutes.

f. An automatic plate washer is used to aspirate all wells of the plate. The plate is washed three times with 300 μl wash solution/well.

g. The MAb is diluted in blocker solution to the current use dilution according to the insert instructions. A 100-μl amount

of diluted MAb is added to each well. The plate is incubated at 37°C for 1 hour ± 15 minutes.

h. An automatic plate washer is used to aspirate all wells of the plate. The plate is washed three times with 300 µl wash solution/well.

i. A 100-µl amount of conjugate diluted to the appropriate dilution (approximately 1:1000) in blocker solution is added to each well. The plate is incubated at 37°C for 1 hour ± 15 minutes.

j. An automatic plate washer is used to aspirate all wells of the plate. The plate is washed three times with 300 µl wash solution/well.

k. Substrate is prepared just prior to use. A 100-µl amount of substrate is added to each well. The plate is incubated at room temperature (22-25°C) until color is developed.

l. If TMB is used as the substrate, 100-µl of stop solution is added to each well after 5-15 minutes incubation. No stop solution is used with ABTS.

m. The plates are briefly shaken immediately prior to reading, and plates are read at a dual absorbance of 450/650 nm, 405/650 nm, 450/630, or 450/620 on a microplate reader.

n. The mean optical density (O.D.) for all blank wells is determined, and that value is subtracted from all sample O.D. values prior to data analysis.

D. DATA PROCESSING AND INTERPRETATION

1. Relative potency calculation method

The current version of the National Veterinary Services Laboratories Relative Potency Calculation Program SAM 318 is used to calculate the relative potency of the test serial as compared to that of the reference bacterin. The reference and test serial data are entered, and the program is executed as outlined in SAM 318. The relative potency (RP) value reported for the test serial will be the average of the highest RP values included in the top scores from each replicate.

2. Requirements for a valid assay

An assay must meet the validity requirements of SAM 318 (current version) to be considered valid.

a. Lines determined by first-order linear regression must have a correlation coefficient (r) ≥ 0.95.

b. The reference and test serial lines must show parallelism (slope ratio ≥ 0.80).

Assays that are not valid may be repeated up to a maximum of three times. If a valid assay cannot be achieved with three independent assays, the serial will be reported as unsatisfactory.

3. Requirements for a satisfactory serial

To be considered satisfactory, a test serial must have an RP value of ≥ 1.0.